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Pex8p, an Intraperoxisomal Peroxin of *Saccharomyces cerevisiae* Required for Protein Transport into Peroxisomes Binds the PTS1 Receptor Pex5p*

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We report the characterization of ScPex8p, which is essential for peroxisomal biogenesis in *Saccharomyces cerevisiae*. Cells lacking Pex8p are characterized by the presence of peroxisomal membrane ghosts and mislocalization of peroxisomal matrix proteins of the PTS1 and PTS2 variety to the cytosol. Pex8p is tightly associated with the lumenal face of the peroxisomal membrane. Consistent with its intraperoxisomal localization, Pex8p contains a peroxisomal targeting signal 1, and it interacts with the PTS1 receptor Pex5p. However, the Pex5p/Pex8p association is also observed upon deletion of the PTS1 of Pex8p, suggesting that Pex8p contains a second binding site for Pex5p. The *pex8*Δ mutant phenotype and the observed PTS1-independent interaction with the PTS1 receptor suggest that Pex8p is involved in protein import into the peroxisomal matrix. In *pex8*Δ cells, the PTS1 and PTS2 receptor still associate with membrane bound components of the protein import machinery, supporting the assumption that the Pex8p function in protein translocation follows the docking event.

The sorting of proteins to distinct subcellular compartments is achieved by the coordinated action of organelle-specific targeting signals and receptors. Proteins destined for the peroxisomal matrix are synthesized on free polyribosomes and are imported posttranslationally in pre-existing organelles (1). Two well characterized peroxisomal targeting signals (PTS1 and PTS2)[1] are responsible for the sorting of matrix proteins to the organelle (for review see Refs. 2–4). The PTS1 sequence consists of the C-terminal tripeptide Ser-Lys-Leu and species-specific variations (for review see Ref. 5). The PTS2 is an N-terminal stretch of nine amino acids with the consensus sequence RLX_5(H/Q)L and thus far is found in only a few peroxisomal proteins (for review see Refs. 6 and 7). Recognition of PTS1 is performed by the tetratricopeptide repeat family protein, Pex5p, whereas the PTS2 is recognized by the WD40 protein Pex7p (for review see Refs. 8 and 9). Cells deficient in either of the two proteins display partial import deficiencies: *pex5*Δ cells correctly import PTS2 proteins but are affected in the import of PTS1 proteins, and *pex7*Δ cells exhibit the reverse phenotype (for review see Ref. 10). The intracellular localization of both targeting signal receptors is still a matter of debate. A predominantly cytosolic, membrane-bound, or even intraperoxisomal localization has been reported for either of them (for review see Ref. 3). An attractive model that reconciles the different localization of the import receptors is the "extended shuttle hypothesis" (9, 11, 12). This model is a modification of the original "hypothesis of shuttling receptors" (13), and it suggests that the import receptors Pex5p and Pex7p bind their cargo proteins in the cytosol, dock to specific proteins at the periphery of the peroxisomal membrane, subsequently enter the peroxisome, release their cargo in the peroxisomal lumen, and shuttle back to the cytoplasm. The observation that peroxisomes are capable of importing folded and oligomeric proteins is in line with this model (for review see Ref. 5).

So far, two peroxisomal membrane proteins have been described that display the necessary properties to serve as peroxisomal docking sites for the PTS receptors. Pex13p, an integral peroxisomal membrane protein, specifically binds by means of its cytosolic SH3 domain to the PTS1 receptor Pex5p (14–16). Recently, it has been reported that Pex13p also interacts with the PTS2 receptor and that the protein is required for the peroxisomal localization of Pex14p (17). In yeast, Pex14p is a peripheral membrane protein located at the cytosolic side of the membrane outer face of the peroxisome (18–20), and the protein has been shown to physically interact with both receptors, Pex5p and Pex7p, as well as with the peroxisomal membrane proteins Pex13p and Pex17p (18, 21). It has been proposed that the two protein import pathways are not independent but overlapping with Pex13p or Pex14p being the point of convergence of the pathways at a common translocation site for matrix proteins at the peroxisomal membrane (17, 18).

Here, we describe the cloning of the *Saccharomyces cerevisiae PEX8* gene by functional complementation of the peroxisome biogenesis mutant *pex8-1*, which is characterized by the inability to grow on fatty acids as single carbon source, the absence of morphologically detectable peroxisomes, and the mislocalizaion of peroxisomal matrix proteins to the cytosol. We also identified the *PEX8* gene product by sequence determination of isolated peroxisomal membrane proteins. The ScPex8 protein shows significant sequence similarity to three proteins of other yeasts that were shown to be required for

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[1] The abbreviations used are: PTS, peroxisomal targeting signal; HPLC, high pressure liquid chromatography; kb, kilobase; PAGE, polyacrylamide gel electrophoresis.

peroxisome biogenesis: HpPex8p (22), PpPex8p (23), and YlPex8p (24). Even though the overall sequence identity between ScPex8p and these proteins is surprisingly low (13–21%), clusters of similarity spread over the entire length of the protein as well as the *pex* phenotype of the corresponding null mutant suggest that the four proteins are orthologues. The Sc*PEX8* gene product is characterized by the presence of a C-terminal PTS1, which, however, is not required for the targeting of Pex8p to peroxisomes. We show that ScPex8p is a peroxisomal membrane protein located at the matrix side of the membrane. Our data indicate that Pex8p is required for import of both PTS1 and PTS2 proteins; however, it seems to be dispensable for the targeting and insertion of peroxisomal membrane proteins. We provide evidence for a direct involvement of Pex8p in protein transport across the peroxisomal membrane, indicated by the fact that the PTS1 receptor Pex5p interacts with Pex8p independent of its C-terminal SKL tripeptide. Taken together, these findings strongly suggest that Pex8p is a component of the peroxisomal transport machinery.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and General Methods—The yeast strains used in this study were *S. cerevisiae* wild-type *UTL-7A* (*MATa, ura3-52, trp1, leu2-3, 112*; W. Duntze, Bochum), *pex8-1* (*MATa, ura3-52, trp1, leu2-3, 112, pex8-1*; this study), *pex8*Δ (*MATa, ura3-52, trp1, pex8:: LEU2*; this study), *pex5*Δ *(MATα, ura3-52, trp1 prb1-112, pep4-3, pex5::LEU2*; Ref. 25), *pex7*Δ (*MATa, ura3-52, trp1 pex7::LEU2*; Ref. 13), *pex13*Δ (*MATa, ura3-52, trp1, pex14::LEU2*; Ref. 15), *pex14*Δ (*MATa, ura3-52, trp1, pex14::LEU2*; Ref. 18), *pex17*Δ (*MATa, ura3-52, trp1, pex14::LEU2*; Ref. 21), *fox3*-2A (*MATa*, *ura3-52*, *trp1*, *leu2-3*/*112*, *ade2*, *fox3*; Ref. 26), HF7c (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3*/*112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17mers)$_3$-CYC1-lacZ*; CLONTECH Laboratories, Inc.), and PCY2 (*MATα*, *gal4*Δ, *gal80*Δ, *URA3::GAL1-lacZ, lys2-801*^{amber}, *his3-*Δ*200, trp1-*Δ*63, leu2 ade2-101*^{ochre}; Ref. 27). Complete and minimal media used for yeast culturing have been described previously (28). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. 0.025 g/liter $CuSO_4$ was added to YNO medium in all experiments that required induction of the *CUP1* promotor (29).

Isolation of the pex8 Mutants—After mutagenesis of UTL-7A cells using ethyl methanesulfonate (30), mutants defective in the growth on oleic acid as single carbon source were isolated (28). Three of these mutant strains fell into the same complementation group designated *pex*8. The screening protocol included replica plating on YNO-agar plates, fractionation of yeast cells, and electron microscopy as described by Erdmann *et al.* (28). Genetic analysis was performed by standard yeast techniques (31).

Isolation and Amino Acid Sequencing of Pex8p—Preparation and high salt extraction of peroxisomal membranes, HPLC separation of peroxisomal membrane proteins, and N-terminal sequence analysis of peroxisomal membrane proteins were performed as described (32).

Cloning and Analysis of the PEX8 Gene—The *pex8-1* mutant was used for cloning *PEX*8 by functional complementation with a yeast genomic library of *S. cerevisiae* in the *Escherichia coli*-yeast shuttle vector YCp50 (33). Transformation of *S. cerevisiae* was carried out by a modified lithium acetate method (34). Leu$^+$ transformants were replicated on YNO-agar plates. One out of about 20,000 transformants was able to grow on medium containing oleic acid as sole carbon source (YNO). Isolation of the complementing plasmid was done as described (31). The complementing plasmid contained a 16.0-kb insert and was designated YCp*PEX8*/16.0. Fragments obtained by *Sau*3AI digestion as well as defined restriction fragments were subcloned into the low copy *CEN4-URA3* plasmid pRS316 (35). The resulting plasmids were tested for complementation by transformation of the *pex8-1* mutant, selection for Ura$^+$, and subsequent screening on YNO-agar plates for oleic acid utilization. pRS*PEX8*/3.2 contained a 3.2-kb genomic *Pvu*II/*Sac*I fragment, which comprised the full complementing activity.

DNA Sequencing—For sequencing, the genomic 3.2-kb *Pvu*II/*Sac*I fragment of plasmid pRSP6/3.2, subclones of defined restriction fragments, and DNaseI deletion fragments were introduced into pBluescript vectors (Stratagene). Sequence analysis was carried out using the dideoxy chain termination method (36). The predicted protein sequence of *PEX8* was used to search EMBL nucleotide sequence data base for similarities with other known protein sequences using the GENEPRO program (Riverside Scientific Enterprise, Seattle, WA) as well as Blast and FASTA programs. Hydropathy analysis was carried out according to the methods of Kyte and Doolittle (37).

Construction of a pex8 Null Allele—For the construction of the *PEX8* gene deletion construct (YEpGD/6), a complementing 3.5-kb *Kpn*I/HindIII fragment of YCp*PEX8*/16.0 was subcloned into YEp352 (38) resulting in YEp*PEX8*/3.5. The *S. cerevisiae LEU2* gene of plasmid pJJ252 (39) was isolated by digestion with *Bam*HI and *Xba*I and used to substitute the internal 1.65-kb *Bam*HI/*Xba*I fragment of the *PEX8* open reading frame leading to YEpGD/6. The *LEU2* gene with flanking regions of *PEX8* was isolated after digestion of YEpGD/6 with *Pst*I and *Hin*dIII and subsequently introduced into wild-type strain UTL-7A. The resultant leucin-prototrophic transformant was crossed to wild-type XDC-10A, the diploid was induced to sporulate, and the meiotic progeny were examined by standard tetrad analysis. Crossing the original mutants with the resultant leucin-prototrophic transformant led to diploid cells that were unable to grow on YNO-agar.

Plasmids—Plasmid YEp105 (40) contains the polyubiquitin gene UBI4 fused downstream of the c-Myc epitope under the control of the inducible *CUP1* promotor (29). To generate myc-*PEX8* fusions, the *Bam*HI/*Kpn*I CUP1mycUb cassette from YEp105 was subcloned into pBluescript SK$^+$ (Stratagene), resulting in plasmid SK/mycUb. Using the *Bgl*II site 3′ of the c-Myc and the vector-derived *Kpn*I site, *UBI4* was replaced by a 2.2-kb *Bam*HI/*Kpn*I *PEX8* fragment, resulting in SK/mycP6. In this c-myc*PEX8* fusion, the first 54 base pairs of *PEX8* open reading frame are replaced by the c-Myc epitope. The c-Myc *PEX8* gene fusion under control of *CUP1* was subcloned in *Bam*HI/*Kpn*I restricted pRS316 (35) and YEp352 (38), resulting in pRSmyc*PEX8*-SKL and YEpmyc*PEX8*-SKL, respectively. The N-terminal amino acid sequence of the fusion protein is as follows: MCEQKLISEEDLGMQIQYDQR-Pex8p.

Mutagenesis of the PTS1 was performed by polymerase chain reaction using the following primers: primer 1 (5′-TATATGAGCTCAGTACTTAATGATACTATAATTT**TC**AAGA-3′) and primer 2 (5′-TATATGAGCTCAGTACTTAATGATACTAT**C**ATTTAGAAGA-3′) (modified nucleotides are in bold type), and T3 sequencing primer (5′-ATTAACCCTCACTAAAG-3′). The template used was a 1.2-kb *Cla*I/*Spe*I fragment of *PEX8* subcloned in pBluescript vector SK. Primers 1 and 2 included the *PEX8 Sca*I site and an additional primer-derived *Sac*I site. Primer 1 was used to substitute the codon for serine by a stop and primer 2 for the substitution of the leucine codon by a stop. Polymerase chain reaction products were subcloned into pBluescript SK$^+$ by the use of the *PEX8* internal *Cla*I and primer-derived *Sac*I sites. Furthermore, the internal *Cla*I/*Sca*I fragment was fused to the 5′ noncoding region of *PEX8 Sca*I/*Kpn*I (of plasmid SK/P6 *Bam*HI/*Hin*dIII) by three-piece ligation into pBluescript SK$^+$. The mutagenized *PEX8* constructs were used to replace the corresponding wild-type fragment by use of the internal *Cla*I and the vector-derived *Kpn*I site of pRSmyc*PEX8* or YEpmyc*PEX8*, respectively. The resulting plasmids were designated pRSmyc*PEX8*ΔSKL (or pRSmyc*PEX8*SKΔL) and YEpmyc*PEX8*ΔSKL.

Other plasmids are specified in the sections describing the isolation of the *PEX8* gene, the two-hybrid analysis and the construction of a *pex8* null allele. Recombinant DNA techniques, including enzymatic modification of DNA, fragment purification, bacterial transformation, and plasmid isolation were performed essentially as described by either Maniatis *et al.* (41) or Ausubel *et al.* (31).

Fractionation of Yeast Lysates and Purification of Peroxisomes—Organelle preparation by differential centrifugation of yeast lysates was performed as described (28). For separation of cell organelles by density gradient centrifugation, cell lysates of wild-type and mutant strains were loaded onto continuous 20–53% (w/w) sucrose density gradients (24 ml). Centrifugation, fractionation of the gradient, and preparation of samples for SDS-PAGE were carried out as described (42). Organellar pellets of oleate-induced wild-type and mutant strains were prepared according to the methods of Erdmann *et al.* (28).

Antibodies and Immunoblots—Electrophoresis and electroblotting onto nitrocellulose was carried out according to standard protocols (43). Anti-thiolase (Fox3p), anti-Pcs60p, anti-Pex3p, anti-Pex14p, anti-Pex13p, and anti-Pex17p antibodies have been described previously (18, 21, 42, 44, 45). Anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase (Amersham Pharmacia Biotech) were used as the second antibody, and blots were developed using the ECL system (Amersham Pharmacia Biotech).

Two-hybrid Analysis—The two-hybrid assay was based on the method of Fields and Song (46). The tested genes were fused to the DNA-binding domain or trans-activating domain of *GAL4* in the vectors pPC86 and pPC97 (27). To generate a *PEX8* construct in pPC97, a

*Bam*HI/*Sca*I genomic fragment was isolated from pRS*PEX8*/3.2 and cloned into pPC97 digested with *Bgl*II and *Sac*I (the *Sac*I site was blunt ended with T4 polymerase). This construct (pPR6/56) encoded a Gal4-Pex8p fusion protein consisting of amino acids 19–589 of Pex8p. To generate PTS1 mutant alleles of *PEX8* in the two-hybrid vector, the 3′ coding region of *PEX8* with the mutation of interest was excised from pRSmyc*PEX8*Δ*SKL* or pRSmyc*PEX8SK*Δ*L*, respectively, using the internal *Xho*I site and the vector *Kpn*I site. These fragments were used to replace the corresponding wild-type portion of *PEX8* in a pUC18 (Promega)-based construct (pUC18*PEX8* that contained a genomic *Bam*HI/*Hin*dIII *PEX8* fragment), again making use of the internal *Xho*I site and the vector-derived *Kpn*I site. The resulting constructs were designated pPR6/78 (encoding for the SKΔL mutation) and pPR6/82 (encoding for the ΔSKL deletion). The inserts of both pPR6/78 and pPR6/82 were subsequently excised with *Bam*HI and *Sac*I (derived from the mutagenesis primers see below) and subcloned into *Bgl*II/*Sac*I-digested pPC97.

Cotransformation of two-hybrid vectors into the strain *PCY2* was performed according to Ref. 34. Transformed yeast cells were plated onto SD synthetic medium without tryptophane and leucine. β-Galactosidase filter assays were performed according to Rehling *et al.* (47). *PEX5* truncation constructs and the luciferase two-hybrid constructs were kindly provided by Henk Tabak (48).

Immunofluorescence and Electron Microscopy—Immunofluorescence microscopy was performed essentially according to Ref. 49 with modifications described (26). CY3-conjugated donkey anti-mouse IgG and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as 6 μg/ml solutions for detection. Potassium permanganate fixation and preparation of intact yeast cells for electron microscopy were performed as described (28).

Membrane Preparation and Protease Protection Assay—Membrane preparation from an organelle pellet enriched for peroxisomes and mitochondria has been described (50). For protease protection assays, an organelle pellet was resuspended in homogenization buffer (28) but without protease inhibitors. Equal amounts were incubated for 30 min on ice with increasing amounts of trypsin. The proteinase was inhibited by the addition of trypsin inhibitor and immediately precipitated with trichloroacetic acid. Samples were subsequently processed for SDS-PAGE.

Co-immunoprecipitation—Immunoprecipitation of Myc-tagged Pex7p from cell lysates was performed as described (7) with the exception that the 35,000 × *g* step was omitted. For immunoprecipitation of Myc-tagged Pex8p from organelles, yeast cells expressing Myc-tagged Pex8p with or without C-terminal tripeptide SKL were grown on 0.3% SD medium to late log phase and, subsequently, for 15 h in YNOG (0.1% glucose, 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract, and 0.67% yeast nitrogene base). Yeast cells were spheroplasted and homogenized, and organelles were sedimented by differential centrifugation at 25,000 × *g* according to Erdmann *et al.* (28). Membrane proteins of the resulting organelle sediments were solubilized with 1.2 ml of buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% digitonin) supplemented with protease inhibitors (0.5 mM NaF, 0.02% phenylmethylsulfonyl fluoride, 15 μg/ml of bestatin, 1.5 μg/ml of pepstatin, 1 μg/ml of leupeptin, 0.1 μg/ml chymostatin). Samples were centrifuged at 200,000 × *g* for 30 min (Sorvall AH650, 40,850 rpm). Supernatants were normalized for protein and volume and incubated with 50 μl of sheep anti-mouse Dynabeads (Dynal, Hamburg, Germany) covered with monoclonal anti-Myc IgG (51) for 2 h at 4 °C. Subsequently, the beads were washed five times for 10 min with 1 ml of buffer A with protease inhibitors, and Dynabead-bound proteins were eluted with 60 μl of SDS-PAGE sample buffer.

For decoration with antibodies, 50 μl of Dynabeads were saturated with monoclonal anti-Myc antiserum at 4 °C overnight. The supernatant was removed, and the beads were washed five times with 1 ml of buffer A and resuspended in 50 μl of buffer A with protease inhibitors.

Analytical Procedures—Acetyl-CoA acyltransferase (3-oxoacyl-CoA thiolase; EC 2.3.1.16), catalase (EC 1.11.1.6), cytochrome *c* oxidase (EC 1.9.3.1), and fumarate hydratase (fumarase; EC 4.2.1.2) were assayed by established procedures (52, 53).

RESULTS

Cloning of the PEX8 Gene and Identification of the PEX8 Gene Product—A mutant strain belonging to an uncharacterized *S. cerevisiae pex* complementation group was identified by its inability to grow on oleic acid as single carbon source and mislocalization of peroxisomal matrix enzymes to the cytosol, consistent with a defect in peroxisome biogenesis (see below).

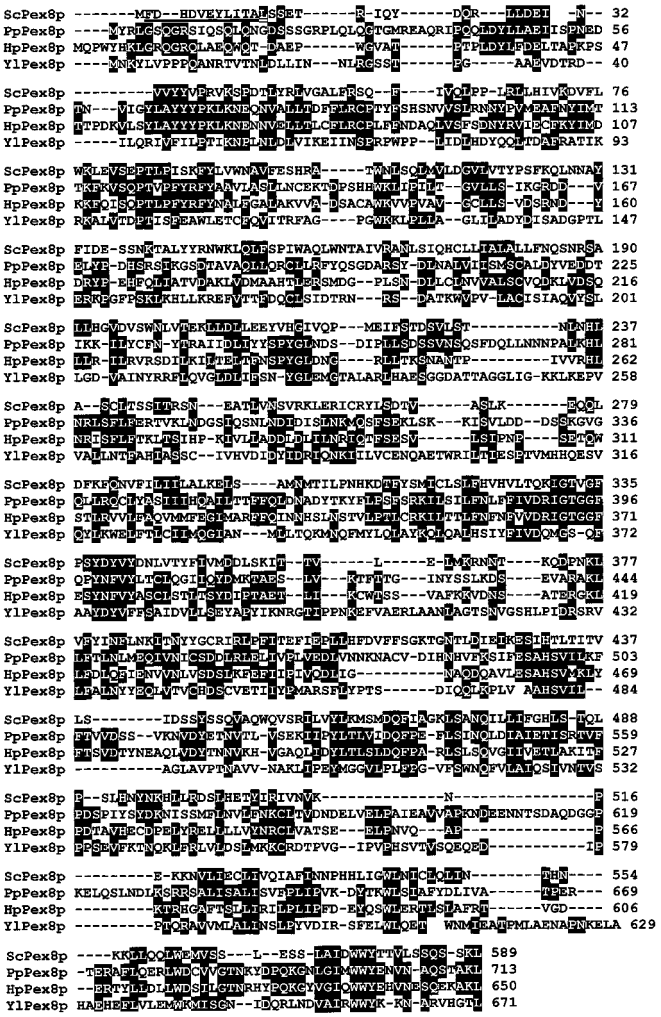

FIG. 1. **Comparison of deduced amino acids of Pex8p of *S. cerevisiae* (ScPex8p), *P. pastoris* (PpPex8p; Ref. 23) *H. polymorpha* (HpPex8p; Ref. 22), and *Y. lipolytica* (YlPex8p; Ref. 24).** Identical amino acids that are present in at least two of the four proteins are indicated. Identity of ScPex8p to PpPex8p, HpPex8p, and YlPex8p is 21, 21, and 13%, respectively. The *underlined* amino acid sequence of ScPex8p was obtained by peptide sequencing of purified Pex8p (Fig. 2).

The meiotic segregation behavior revealed the defect to be caused by a single gene. The diploids obtained by backcrossing of the mutant strain to wild-type cells did not show the mutant phenotype, confirming the mutation to be recessive. The corresponding wild-type gene was cloned by functional complementation of the mutant with a genomic library. Nucleotide sequencing of the smallest complementing insert (3.2-kb *Pvu*II-*Sac*I fragment) revealed an open reading frame of 1,767 base pairs that has also been sequenced in the *S. cerevisiae* genome sequencing project (YGR077c). The open reading frame encodes a hydrophobic protein without obvious transmembrane regions and that has a calculated molecular mass of 68.2 kDa. A search of protein data bases revealed a significant overall amino acid sequence identity between our open reading frame and three proteins from other yeasts (Fig. 1): HpPex8p (formerly Per1p) of *Hansenula polymorpha* (22)), PpPex8p (formerly Per3p) of *Pichia pastoris* (23), and YlPex8p (formerly Pex17p) of *Yarrowia lipolytica* (24), which have previously been shown to be required for peroxisome biogenesis. Even though the overall sequence identity between ScPex8p and these proteins is surprisingly low (13 to 21%), clusters of similarity spread over the entire length of the protein as well as the *pex* phenotype of the

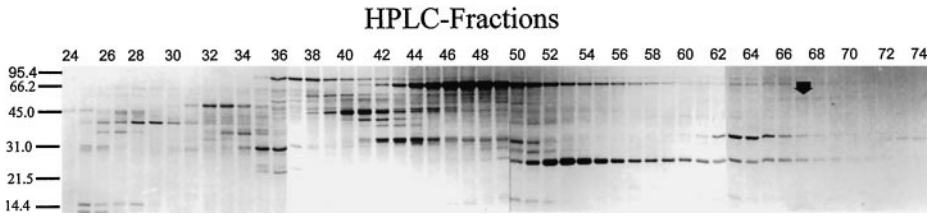

FIG. 2. **Isolation of Pex8p by preparative chromatographic separation of peroxisomal membrane proteins.** High salt-extracted peroxisomal membranes (1 mg of protein) were solubilized in SDS and separated by reverse phase HPLC. Polypeptides of selected fractions were separated by SDS-PAGE and visualized by Coomassie blue staining. The Pex8p band is indicated by the *arrow*. The amount per lane corresponded to 5% of the total fractions. Molecular mass standards (in kDa) are indicated on the *left*.

corresponding null mutant suggest that the four proteins are orthologues. Based on these similarities, the newly identified gene was designated *ScPEX8*.

In parallel, *Sc*Pex8p was identified by a reverse genetic approach. Peroxisomal membranes were prepared from oleate-induced *S. cerevisiae* and successively extracted by low salt and high salt (see "Experimental Procedures"). The membrane proteins were solubilized by SDS and separated by reverse phase HPLC and subsequent SDS-PAGE (Fig. 2). N-terminal sequencing of a high salt-resistant 62-kDa protein of the preparation (indicated by an *arrow* in Fig. 2) gave the peptide sequence FDHDVEYLITAL that matched Pex8p. The comparison of the Coomassie-stained proteins in Fig. 2 indicates that Pex8p is a protein of very low abundance as typical for most yeast peroxins. Pex8p is one of the last peroxisomal proteins eluting from the butyl-column that is in agreement with the extremely hydrophobic nature of the protein (Fig. 2).

The deduced Pex8p sequence did not contain any characteristic features that would allow a prediction of its function. Using the PROSITE data base, a heptad leucine-zipper motive was identified (amino acid positions 253–274). The most interesting sequence feature of Pex8p was the presence of a peroxisomal targeting signal 1 (PTS1, Ser-Lys-Leu) at the extreme C terminus of the protein (Fig. 1). This PTS1 is known to be sufficient to target proteins into microbodies in virtually all eukaryotes. Moreover, there was an internal amino acid stretch (amino acids 103–112) that resembled the proposed consensus sequence for a PTS2 (6, 7).

pex8Δ Cells Exhibit an Import Defect for Peroxisomal Matrix Proteins—A *PEX8* deletion mutant (*pex8Δ*) was generated by replacing base pairs 59–1706 of the *PEX8* open reading frame by *LEU2*. Functional complementation studies of backcrosses of *pex8Δ* with the original *pex8-1* mutant indicated that both mutants are allelic (data not shown). Cells deficient in *PEX8* are viable on YPD, SD, and ethanol media, but they are unable to grow on oleic acid as single carbon source (Fig. 3*A*), typical for peroxisomal mutant strains of *S. cerevisiae* (28). Growth on oleic acid medium was restored upon transformation with a single copy plasmid harboring the *PEX8* gene (Fig. 3*A*).

The ultrastructure of oleic acid-induced *pex8Δ* mutant cells revealed that Pex8p is involved in the biogenesis of peroxisomes. In oleic acid-induced wild-type cells, the peroxisomes are easily recognized by their characteristic electron-dense matrix (Fig. 4*A*). Cells lacking Pex8p are characterized by the absence of morphologically recognizable peroxisomes (Fig. 3*B*), which were restored upon transformation of the mutant cells with the *PEX8* gene (Fig. 4, *C* and *D*).

The involvement of Pex8p in peroxisome biogenesis is also supported by the mislocalization of peroxisomal matrix proteins to the cytosol as observed by organelle sedimentation analysis (Fig. 3*B*). The subcellular distribution of the peroxisomal matrix enzymes catalase, thiolase (Fox3p), and the multifunctional enzyme of the peroxisomal β-oxidation system as well as mitochondrial fumarase were determined by cell fractionation analysis of wild-type, *pex8-1*, and *pex8Δ* cells. Organelles of oleic acid-induced cells were separated by differential centrifugation, and peroxisomal and mitochondrial marker enzyme activities of the sediment and supernatant fractions were determined (Fig. 3*B*). In wild-type cells, the majority of the peroxisomal and mitochondrial enzymes were detected in the organelle pellet. However, in *pex8-1* and *pex8Δ* cells, the peroxisomal matrix proteins were predominantly found in the soluble fraction, consistent with their mislocalization to the cytosol. The mislocalization of peroxisomal thiolase and cata-

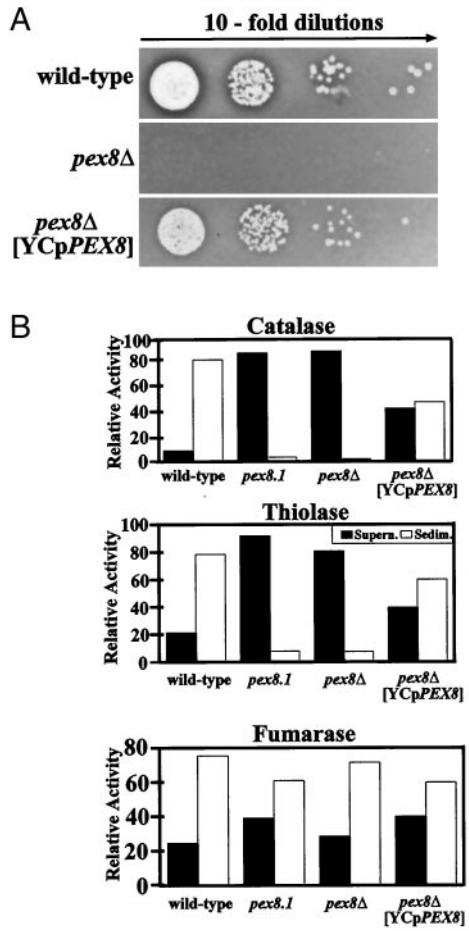

FIG. 3. **Cells lacking Pex8p are characterized by an inability to grow on oleic acid containing medium and mislocalization of peroxisomal marker enzymes to the cytosol.** *A*, growth on oleic acid medium of wild-type, *pex8Δ*, and *pex8Δ* cells harboring the *CEN*-plasmid YCp*PEX8*. *B*, subcellular distribution of peroxisomal and mitochondrial marker enzymes in oleic acid-induced wild-type, *pex8.1*, and *pex8Δ* cells as well as *pex8Δ* cells complemented with YCp*PEX8*. After centrifugation of cell homogenates from oleic acid-induced cells at 25,000 × *g*, the sediments and supernatants were assayed for peroxisomal catalase and thiolase as well as for mitochondrial fumarase activities.

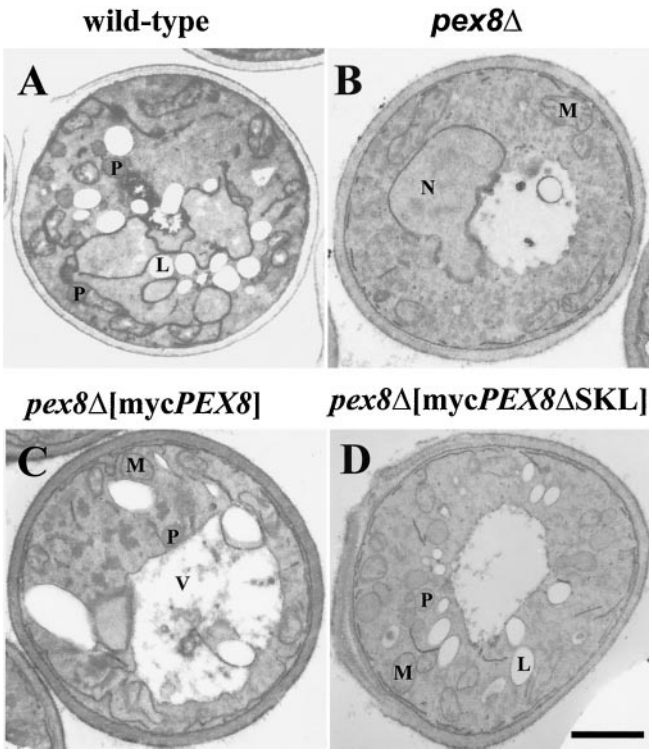

FIG. 4. **Mutant *pex8*Δ cells lack morphologically detectable peroxisomes.** Electron micrographs of oleic acid induced cells of wild-type (*A*), null mutant *pex8*Δ (*B*), and *pex8*Δ cells expressing either myc*PEX8* (*C*) or myc*PEX8*ΔSKL (*D*) from single copy plasmids. Expression of myc*PEX8* or myc*PEX8*ΔSKL results in the functional complementation of the mutant phenotype, indicated by the presence of peroxisomes. *L*, lipid droplet; *M*, mitochondrion; *N*, nucleus; *P*, peroxisome; *V*, vacuole. *Bar*, 2 μm.

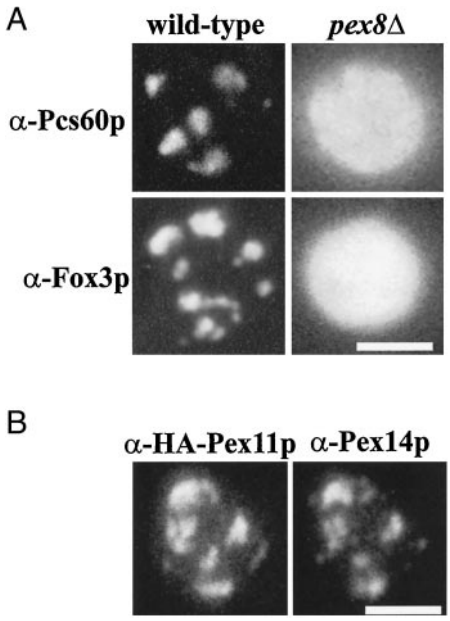

FIG. 5. **Mutant *pex8*Δ cells are defective in peroxisomal matrix protein import but not in the formation of the peroxisomal membrane.** *A*, immunofluorescence microscopy localization of the PTS1 protein Pcs60p and the PTS2 protein Fox3p in wild-type and *pex8*Δ mutant cells. The punctate pattern indicates a peroxisomal labeling of these proteins in wild-type cells. The overall fluorescence observed for the *pex8*Δ mutant indicates the mislocalization of the proteins to the cytosol. Oleic acid-induced wild-type and *pex8*Δ cells were processed for immunofluorescence microscopy using polyclonal rabbit-antibodies against Pcs60p and Fox3p as indicated. Secondary antibodies were fluorescein isothiocyanate-conjugated anti-rabbit IgG. *Bar*, 5 μm. *B*, double immunofluorescence microscopy localization in a *pex8*Δ cell of the peroxisomal membrane marker HA-Pex11p (32, 57) and Pex14p (18, 19), a peripheral component of the yeast peroxisomal protein import machinery. Oleic acid-induced *pex8*Δ cells were processed for double immunofluorescence microscopy using monoclonal mouse-antibodies against HA-Pex11p and polyclonal rabbit antibodies against Pex14p as indicated. Each panel shows a single cell, and secondary antibodies were CY3-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG, respectively. The congruent punctate pattern for the localization of both proteins indicates the presence of Pex14p-containing peroxisomal membrane ghosts in *pex8*Δ cells. *Bar*, 5 μm.

lase was complemented by transformation of the mutant cells with a plasmid harboring the *PEX8* gene.

The peroxisomal import defect of *pex8*Δ mutant cells was also corroborated by immunofluorescence microscopy localization of peroxisomal marker enzymes (Fig. 5*A*). Wild-type cells exhibit a peroxisome characteristic punctate pattern when stained for the PTS1 protein Pcs60p (45) or the PTS2 protein thiolase (Fox3p) (26, 54). In contrast, a diffuse staining pattern for both peroxisomal matrix proteins is observed in *pex8*Δ cells, indicating their mislocalization to the cytosol. These data demonstrate that *pex8*Δ cells exhibit an import defect for peroxisomal matrix proteins of the PTS1 variety as well as the PTS2 variety.

Import of peroxisomal matrix proteins and integration of peroxisomal membrane proteins occur by different pathways (15, 16). If Pex8p is involved only in the topogenesis of peroxisomal matrix proteins, the targeting of peroxisomal membrane proteins is expected to remain unaffected in *pex8*Δ cells. Indeed, double immunofluorescence localization of the peroxisomal membrane marker Pex11p and the matrix marker Pcs60p or in *pex8*Δ cells revealed a localization of Pcs60p throughout the cytosol, whereas a peroxisome characteristic punctate pattern was observed for Pex11p. Moreover, double immunofluorescence localization of Pex11p and Pex14p in *pex8*Δ cells revealed a congruent punctate fluorescence pattern, suggesting co-localization of both proteins on peroxisomal membrane ghosts in *pex8*Δ cells (Fig. 5*B*). The presence of peroxisomal membrane ghosts in *pex8*Δ cells indicates that Pex8p is involved in the topogenesis of peroxisomal matrix proteins but is not required for the targeting of peroxisomal membrane proteins.

Subcellular Localization of Pex8p—Attempts to localize the endogenous Pex8p with anti-Pex8p antibodies failed, probably because of the very low abundance of the protein (Fig. 2). To analyze the subcellular distribution of Pex8p, we made use of an N-terminally Myc-tagged Pex8p. In this fusion protein, the first 18 amino acids of Pex8p are replaced by the Myc epitope (51). Expression of the fusion protein was under the control of the *CUP1* promoter (29). Expression of the fusion protein plasmids did result in functional complementation of the *pex8*Δ growth defect on oleic acid medium (Fig. 6), reappearance of morphologically detectable peroxisomes (Fig. 4*C*), and restoration of the peroxisomal localization of Fox3p (Fig. 7, *A* and *B*). This result indicated that the tagged Pex8p is functionally active. Thus, its subcellular localization could be expected to closely mirror the localization of the endogenous Pex8p. Double immunofluorescence microscopy localization of the Myc-tagged Pex8p and the peroxisomal marker Fox3p revealed a congruent punctate fluorescence pattern (Fig. 7*A*). This result suggests that Pex8p is peroxisomal. However, a perfect co-localization was mostly observed in cells that did show a low expression level of the tagged Pex8p. Cells that showed a brighter fluorescence because of a high expression of mycPex8p did frequently exhibit Pex8p containing spots that did not co-localize with Fox3p (data not shown). Because expression of the Myc-tagged Pex8p was under the control of a strong promoter, the additional labeling is likely due to an aggegation of mislocalized Pex8p. In sucrose density gradients of homogenates from oleic

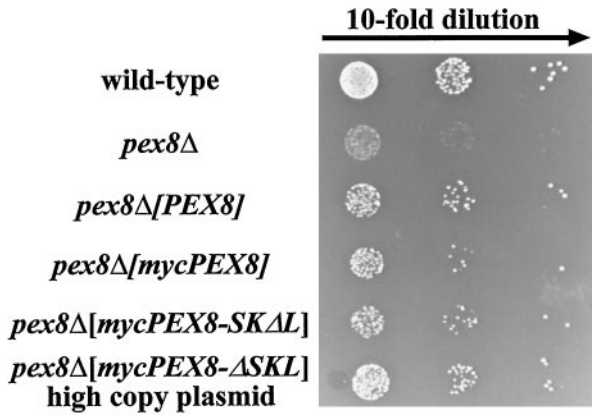

FIG. 6. **Functional complementation of *pex8*Δ cells with Myc-tagged Pex8p independent of the presence of the PTS1.** *pex8*Δ cells were transformed with low copy plasmids pRS*PEX8*, pRSmyc*PEX8*, pRSmyc*PEX8SKΔL*, or the episomal plasmid YEpmyc*PEX8ΔSKL* as indicated. Expression of the latter two plasmids leads to the synthesis of mycPex8p lacking either the leucine of the C-terminal tripeptide PTS1 (*PEX8SKΔL*) or lacking the entire PTS1 (*PEX8ΔSKL*). Expression of all constructs did result in a functional complementation of the oleic acid growth defect of *pex8*Δ cells, indicating that neither the tagging nor the mutations in the PTS1 significantly affected the function of Pex8p in peroxisome biogenesis. Cells were grown on oleic acid medium for 5 days at 30 °C.

acid induced, complemented *pex8*Δ(myc*PEX8*) cells, mycPex8p comigrated with the peroxisomal marker enzyme catalase at a density of 1.22 g/ml (Fig. 7*B*), indicative of a peroxisomal localization of mycPex8p. However, a significant amount of mycPex8p is found in fractions 19–23 at lighter densities. These portions might represent the putative Pex8p aggregates, which were also observed by immunofluorescence microscopy.

To analyze the subperoxisomal localization of Pex8p, an organellar fraction isolated from spheroplasts of complemented *pex8*Δ(myc*PEX8*) cells was subjected to extraction by 10 mM Tris, pH 8.0, and subsequently with 100 mM Na_2CO_3 buffer, pH 11.5. Pex8p was resistant to low salt extraction but could be completely released from membranes by treatment with carbonate (Fig. 7*C*). These extraction properties distinguished Pex8p from two other peroxisomal proteins. Pex3p (42) was resistant to either means, consistent with its being an integral membrane protein (Fig. 7*C*). Peroxisomal thiolase (Fox3p) (44) was already extracted by the treatment with the hypotonic low salt solution, as expected for a protein localized in the peroxisomal matrix. Thus, in resisting low salt extraction but succumbing to carbonate treatment, Pex8p fulfills the requirements for a tightly bound peripheral membrane protein.

Because Pex8p possesses the PTS1 at its C terminus, we presumed that the protein is localized within the peroxisome. This hypothesis was experimentally addressed by means of a protease protection experiment in which a crude organelle pellet isolated from *pex8*Δ cells that expressed the mycPex8p fusion protein was subjected to trypsin treatment in the presence or absence of detergent (Fig. 7*D*). Thiolase, as an intraperoxisomal protein was protected against the protease but was completely degraded upon addition of detergent. In contrast, the Pex3p with its cytoplasmic domain was completely degraded independent of the presence of detergent. Pex8p was protected against trypsin in the absence of detergent, but when detergent was added to the organelles, Pex8p was rapidly degraded. This degradation is most likely due to the presence of endogenous proteases within the organelle pellet, which get access to Pex8p upon addition of Triton X-100. This result suggests that mycPex8p is protected against proteases by the peroxisomal membrane.

The PTS1 of Pex8p Is Not Essential for Its Function in Peroxisome Biogenesis—At present it is a generally accepted view that a PTS1 directs a protein into the peroxisomal matrix, and there is no reason to believe that Pex8p is an exception. This argument and our results concerning the subcellular localization of Pex8p (see above) suggested to us that Pex8p is an intraperoxisomal protein. Therefore, we asked whether the C-terminal SKL of Pex8p is necessary to target Pex8p into the peroxisomal matrix. To address this question, Myc-Pex8p fusion constructs were generated with either partially or completely deleted SKL (mycPex8p-SKΔL and mycPex8p-ΔSKL). *pex8*Δ cells expressing these constructs from either low or high copy plasmids were analyzed for their ability to grow on oleic acid medium. As shown in Fig. 6, all transformants regained the ability to grow on oleic acid plates. There was no significant difference in growth between the transformants independent of the presence or absence of the PTS1. Moreover, electron microscope analysis demonstrated that *pex8*Δ cells expressing mycPex8p-SKΔL, mycPex8p-ΔSKL, or mycPex8p-SKL (with original C terminus) possess peroxisomes that were morphologically indistinguishable from those of wild-type cells (Fig. 4). This unexpected observation indicates that the presence of the PTS1 of Pex8p seems not to be essential for the function of the protein. Moreover, the subcellular localization of mycPex8p-ΔSKL was analyzed by sucrose density gradient centrifugation of cell lysates from transformants (Fig. 8). Cosegregation of mycPex8pΔSKL with catalase in fractions of 1.23 g/cm to 1.21 g/cm suggest that the PTS1-deficient Pex8p is still peroxisomal. We conclude that the truncated protein had reached its functional site in a PTS1-independent manner.

Pex8p Interacts with the PTS1 Receptor—To date Pex8p is one of 19 peroxins that have been shown to be involved in peroxisome biogenesis in *S. cerevisiae*. There is striking evidence that many of these peroxins interact during their function in peroxisome biogenesis (9). Therefore, we used the two-hybrid system to detect putative binding partners for Pex8p (46, 55). Fusion constructs were prepared by cloning *PEX* genes into plasmids encoding either the activation or DNA-binding domains of Gal4p. Physical interaction of Pex8p with peroxins was expected to result in the activation of *lacZ* transcription of transformants. Yeast cells co-expressing Pex8p and Pex5p (25, 56) fused to the corresponding Gal4p domains expressed significant amounts of β-galactosidase, demonstrating that Pex8p is capable of binding to the PTS1 receptor Pex5p *in vivo* (Fig. 9*A*). To determine whether this interaction just reflects the binding of Pex5p to the PTS1 of Pex8p, fusion constructs of either full-length Pex8p or Pex8pΔSKL with the Gal4p-DB domain were tested against Gal4p-AD-Pex5p fusions. Double transformants of the yeast reporter strain PCY2 and HF7c were tested for reporter gene activation by assaying for β-galactosidase activity (Fig. 9*A*) or histidine auxotrophy (Fig. 9*B*), respectively. Both Pex8p with and without SKL were found to associate with full-length Pex5p. Thus, the SKL tripeptide of Pex8p is not required for the two-hybrid interaction with Pex5p. We also analyzed C-terminal truncation constructs of Pex5p for their ability to bind Pex8p or the PTS1-containing luciferase (Fig. 9*A*). As Pex8p, the luciferase was found to interact with full-length Pex5p. The C-terminal truncation of Pex5p did result in a reduction of the Pex8p-Pex5p two-hybrid interaction; however, both Pex8p with and without SKL significantly bound to truncated Pex5p fusion constructs. In contrast, the PTS1 protein luciferase was only able to bind to the full-length Pex5p. These data indicate that the Pex5p/Pex8p interaction involves regions of Pex5p different from the binding site for the PTS1. These observations are in agreement with the assumption that the observed Pex5p/Pex8p interaction might not just reflect the binding reactions between a cargo protein and its receptor but that the Pex5p/Pex8p interaction

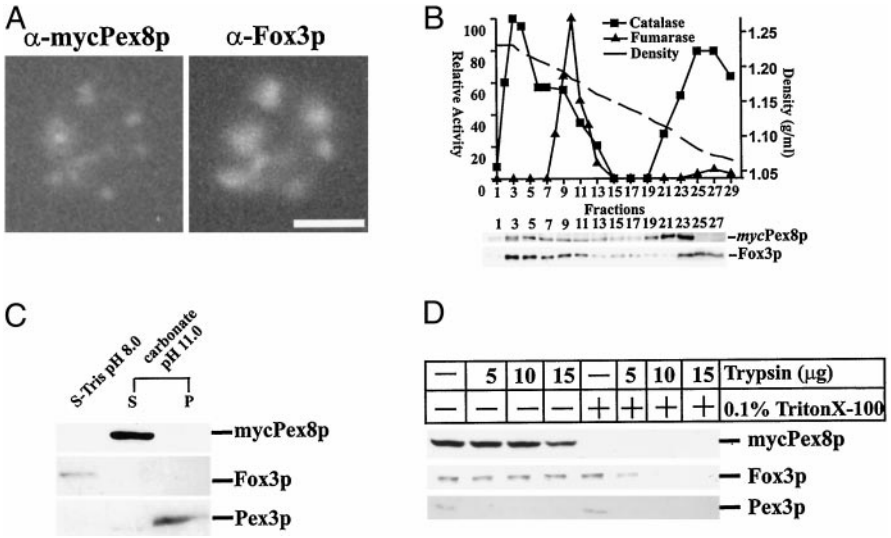

FIG. 7. **Subcellular localization of mycPex8p.** *A*, double immunofluorescence localization of Myc-tagged Pex8p and peroxisomal Fox3p. Oleic acid-induced *pex8*Δ cells complemented by the expression of myc*PEX8* were processed for double immunofluorescence microscopy with monoclonal antibodies against the Myc tag and polyclonal rabbit anti-Focx3p antibodies. The congruent fluorescence pattern observed for mycPex8p and Fox3p suggests that mycPex8p is peroxisomal. Moreover, the punctate pattern observed for the localization of Fox3p is indicative of the restoration of the import capacity of the *pex8*Δ peroxisomes and demonstrates the complementing activity of the Myc-tagged Pex8p. Expression of myc*PEX8* was from a single copy plasmid (pRSmyc*PEX8*). Secondary antibodies were CY3-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG, respectively. *Bar*, 5 μm. *B*, immunological detection of mycPex8p in fractions obtained by isopycnic 20–54% sucrose density gradient centrifugation of cell-free homogenates from oleic acid-induced *pex8*Δ cells expressing mycPex8p from pRSmyc*PEX8*. Peroxisomal marker enzyme catalase as well as mitochondrial cytochrome *c* oxidase were monitored by activity measurements. Equal volumes of each fraction were immunological analyzed for the presence of mycPex8p. *C*, mycPex8p is tightly associated with the peroxisomal membrane. An organellar fraction isolated from *pex8*Δ cells complemented with myc*PEX8* was successively extracted with 10 mM Tris, pH 8.0, and 100 mM Na_2CO_3, pH 11.5. Equal amounts of the extracts (*S*) and membrane fraction (*P*) were separated by SDS-PAGE and analyzed by Western blot using antibodies against Myc epitope, thiolase (Fox3p), and Pex3p. Pex8p was resistant to low salt treatment but was completely released from membranes by the Na_2CO_3 treatment, characteristics that are typical for a peripheral membrane localization. *D*, protease protection analysis of an organellar fraction isolated from spheroplasts of *pex8*Δ cells complemented with myc*PEX8*. Equal amounts of an organellar fraction (200 μg of protein) were incubated for 30 min on ice with increasing amounts of trypsin in the presence or absence of detergent. Samples were analyzed by SDS-PAGE and Western blot using antibodies against the Myc epitope, thiolase (Fox3p), and Pex3p. Pex8p was protected against trypsin in the absence of detergent but was already completely degraded upon addition of detergent, suggesting that Pex8p is an intraperoxisomal protein.

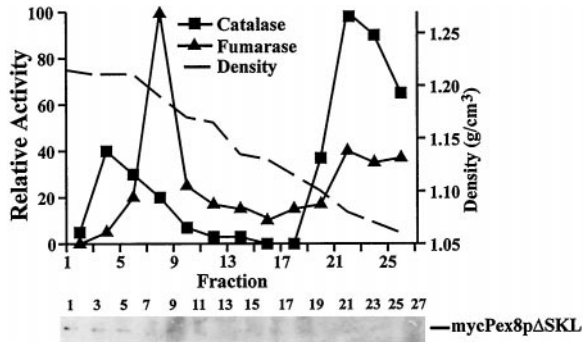

FIG. 8. **Subcellular localization of mycPex8p lacking the PTS1.** Immunological detection of myc*PEX8*Δ*SKL* in fractions that were obtained by isopycnic 20–54% sucrose density gradient centrifugation of cell-free homogenates from oleic acid-induced *pex8*Δ cells expressing myc*PEX8*Δ*SKL* from high copy plasmid (YEpmyc*PEX8*Δ*SKL*). Peroxisomal marker enzymes catalase and thiolase, as well as mitochondrial fumarase, were monitored by activity measurements. Equal volumes of each fraction were immunologically analyzed for the presence of mycPex8p.

might be of importance for the function of these proteins in peroxisome biogenesis. The Pex8p interaction with Pex5p was independently confirmed by co-immunoprecipitation (Fig. 10). Pex5p could be co-immunoprecipitated with Myc-tagged Pex8p from sedimented organelles of transformants expressing the fusion protein but not from control strains. Co-precipitation of Pex8p and Pex5p was independent of the presence of the C-terminal PTS1 of Pex8p. This result further supports the notion that the observed interaction does not simply reflect the PTS1 signal recognition by Pex5p but that the Pex5p/Pex8p association might be of importance for the function of both proteins in PTS1-dependent protein import into peroxisomes.

Pex8p Is Not Required for Import Receptor Docking—The peroxins Pex13p, Pex14p, and Pex17p have been suggested to form a complex at the peroxisomal membrane, which is required for the docking of the import receptors Pex5p and Pex7p. In line with this assumption, these peroxins have been demonstrated to efficiently co-precipitate with Pex7p (18, 21). To determine whether Pex8p is required for the association of this complex, we analyzed its constituents in Pex7p precipitates from *pex8*Δ cells (Fig. 11). The amount of partners for Pex7p in the precipitate from *pex8*Δ cells and from wild-type cells was nearly indistinguishable. Obviously, Pex7p and Pex5p are associated with components of the peroxisomal translocation machinery in the absence of Pex8p, suggesting that the presence of Pex8p is not a prerequisite for docking of Pex7p or Pex5p to the peroxisomal membrane. This observation is in agreement the assumption that Pex8p might function after the docking event, which is also supported by the intraperoxisomal localization of Pex8p. The increased amount of thiolase association with Pex7p in *pex8*Δ cells (Fig. 11) was not surprising because this phenomenon is frequently observed in cells defective in the peroxisomal import of PTS2-containing proteins (17). This observation could be indicative of a defect in cargo release from its receptor; however, the simplest explanation is that the increased association is due to an increased concentration of cytosolic thiolase caused by the import defect.

DISCUSSION

Here we report on Pex8p, a novel peroxin of *S. cerevisiae* that we identified by a forward as well as by a reverse genetic screening approach. The genetic approach aimed to identify

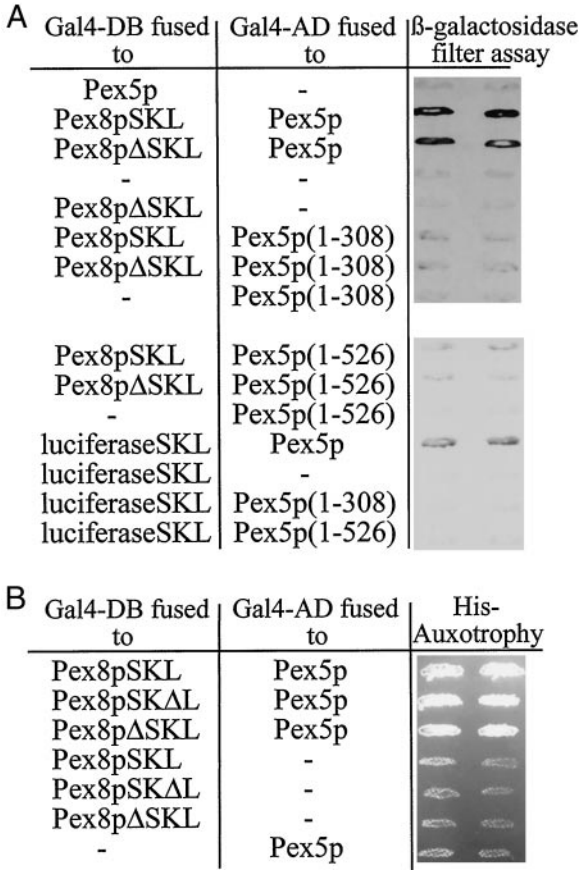

FIG. 9. **PTS1-independent two-hybrid interaction of Pex8p and Pex5p.** *A*, PCY2 double transformants expressing the indicated fusion protein combinations of Pex5p with luciferase or Pex8p with/without PTS1 were analyzed for β-galactosidase activity by a filter assay using X-gal as a substrate. *B*, HF7c double transformants co-expressing Pex5p as well as Pex8p containing the PTS1 of the indicated C-terminal truncations were assayed in the two-hybrid system by means of *HIS3* activation. Transformants were grown on selective plates lacking leucine, tryptophane, and histidine but containing 10 mM 3-aminotriazole. Two representative independent double transformants are shown.

mutant cells that lost their ability to grow with oleic acid as sole carbon source. The second screen used partial protein sequences obtained from purified peroxisomal membrane proteins to screen the yeast genome sequence data base for matching proteins (Fig. 2). Recently, ScPex8p has also been identified in silico by context-sensitive motif searches (57). We cloned the *PEX8* gene by complementation of the original *pex8-1* mutant and subsequently performed a molecular characterization of the Pex8p protein. Analysis of the original *pex8-1* mutant as well as of the *pex8*Δ mutant revealed that these cells are unable to grow on oleic acid as single carbon source (Fig. 3; see Ref. 57); they lack morphologically detectable, mature peroxisomes (Fig. 4) and are characterized by mislocalization of peroxisomal matrix enzymes containing PTS1 and PTS2 variety to the cytosol (Figs. 3 and 5; see Ref. 57). These biochemical, electron microscopical, and immunofluorescence data indicate that *pex8-1* as well as *pex8*Δ cells are affected in protein transport of peroxisomal matrix proteins. Further support for the function of Pex8p comes from findings in other yeast species. The ScPex8 protein shows significant sequence similarity to three proteins of other yeasts that were shown to be required for peroxisome biogenesis: HpPex8p (22), PpPex8p (23), and YlPex8p (24). Even though the overall sequence identity between these proteins is surprisingly low (13–21%), there are clusters of similarity spread over the entire length of the proteins (Fig. 1). Yeast cells lacking either of these proteins are characterized by the lack of morphologically detectable peroxisomes and mislocalization of peroxisomal matrix proteins to the cytosol. The sequence similarity and the similarity in phenotype of the corresponding mutants with respect to their involvement in peroxisome biogenesis makes it likely that the Pex8p proteins from the different species represent true orthologues.

Recently, it has been reported that peroxisomal matrix and membrane proteins are directed to peroxisomes by distinct pathways (15, 16). In line with this assumption, the transport and insertion of most peroxisomal membrane proteins is not affected in mutant cells with a defect in components of the import machinery for peroxisomal matrix proteins (15, 16, 18, 21). Large multimembraned structures and small vesicular structures were observed in *pex8*Δ cells from *P. pastoris* and *H. polymorpha*, respectively (22, 24), but it was not clear whether the observed structures represent peroxisomal membranes. Our data indicate that the peroxisomal membrane marker Pex11p (32, 58) localizes to peroxisomal membranes in *pex8*Δ cells (Fig. 5*B*). This observation suggests that Pex8p is not involved in formation or inheritance of peroxisomal membranes. An additional important finding is that a component of the peroxisomal protein translocation machinery namely Pex14p (18–20) is actually associated with peroxisomal membrane remnants in *pex8*Δ cells (Fig. 5*B*). Thus, we conclude that these membranes should still have the potential to allow receptor/cargo docking to the organelle. Indeed we could demonstrate in co-immunoprecipitation experiments that a complex of the PTS2 protein thiolase (Fox3p), the PTS1 receptor Pex5p, the peroxisomal membrane proteins Pex14p, Pex13p as well as Pex17p can be isolated together with the PTS2 receptor Pex7p from *pex8*Δ cells and that the coprecipitated amount of the individual proteins was not different compared with other mutants affected in protein import (Fig. 11). Accordingly, in *pex8*Δ cells, the transport of a receptor-cargo protein complex to the peroxisomal membrane as well as its docking to the protein translocation machinery seem not to be affected.

The most striking feature of Pex8p is the presence of a PTS1 targeting signal that is known to target a protein across the peroxisomal membrane into the lumen of the organelle (56, 59, 60). Indeed, subcellular fractionation studies revealed that ScPex8p is peroxisomal and protected against exogenously added protease by a membrane, in agreement with the predicted intraperoxisomal localization (Fig. 7). Successive extraction of isolated organelles indicates that ScPex8p is tightly associated with peroxisomal membranes (Fig. 7*C*). The fact that the protein is completely released upon carbonate treatment favors the idea of its being a tightly associated peripheral membrane protein rather than an integral membrane protein. This result on mycPex8p is also in agreement with the isolation of Pex8p as an high salt-resistant peroxisomal membrane protein (Fig. 2). Further support for the intraperoxisomal localization of Pex8p comes from findings in other yeast species. Immunogold labeling and protease protection analysis demonstrated the intraperoxisomal localization of Pex8p in *H. polymorpha* and *Y. lipolytica*, respectively (22, 24). However, HpPex8p is predominantly localized at the edge of the peroxisome, but as the protein is released by low salt treatment, it has been reported to be localized in the peroxisomal matrix (22). In analogy to our observations for ScPex8p, the orthologues from *P. pastoris* and *Y. lipolytica* have been reported to be tightly associated with the peroxisomal membrane, but they probably are not integral membrane proteins (23, 24).

All four putative orthologues of Pex8p are characterized by the presence of a PTS1 at their C terminus (Fig. 1). We found that this sequence motive seems to be dispensable for the

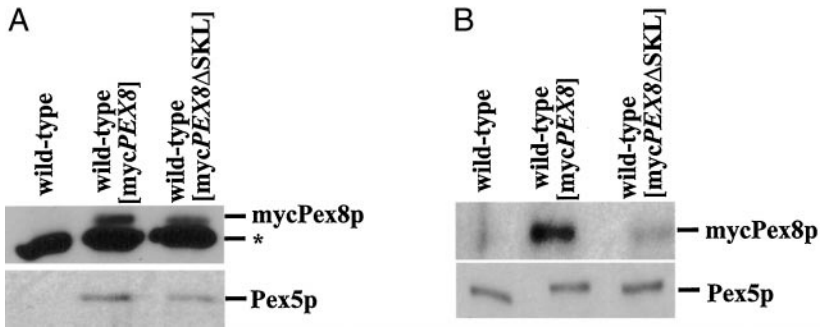

FIG. 10. **mycPex8p is associated with Pex5p even in the absence of C-terminal tripeptide SKL.** *A*, co-immunoprecipitation of mycPex8p and Pex5p. mycPex8p was immunoprecipitated from sedimented membranes of wild-type cells expressing tagged Pex8p with (myc*PEX8*) or without C-terminal tripeptide SKL (myc*PEX8*Δ*SKL*) using antibodies against the c-Myc epitope. Equal amounts of immunoprecipitates (corresponding to 10% of total) were separated by SDS-PAGE and subjected to immunoblot analysis with antibodies against c-Myc and Pex5p. A significant amount of Pex5p co-immunoprecipitated with Pex8p independent of the presence of the PTS1. No Pex5p is found in the precipitate of nontransformed wild-type cells, demonstrating that Pex5p does not unspecifically bind to the IgG-Sepharose column. The *asterisk* indicates the heavy chain of the anti-Myc antibodies that were used for precipitation. *B*, immunoblot analysis showing the total amount of mycPex8p and Pex5p in membrane solubilisates (corresponding to 1% of total) prior to precipitation. The equal amount of Pex5p in the membrane solubilisates indicates that the absence of Pex5p in the wild-type precipitate is not due to a lower amount of Pex5p in the starting material.

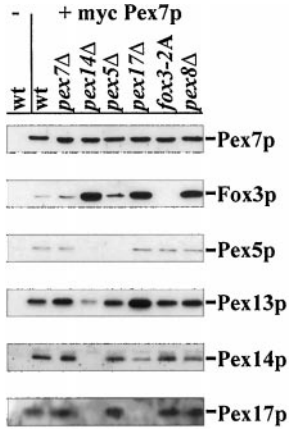

FIG. 11. **Absence of Pex8p does not affect the association of the PTS receptors with the peroxisomal docking complex.** Whole cell extracts of wild-type cells (*wt*) and of wild-type, *pex7*Δ, *pex14*Δ, *pex5*Δ, *pex17*Δ, *fox3-2*A, and *pex8*Δ cells expressing mycPex7p were subjected to immunoprecipitation with monoclonal antibodies against the Myc epitope. Equal amounts of Pex7p immunoprecipitates were separated by SDS-PAGE, and the indicated proteins were detected by immunoblot analysis. The results suggest that Pex8p is not required for the formation of the receptor-docking protein complex, which is in agreement with the assumption that Pex8p might function after the docking event.

function of ScPex8p in peroxisome biogenesis. Our data show that even upon deletion of the PTS1 sequence, Pex8pΔSKL still complements the *pex8*Δ mutant and localizes to peroxisomes of *pex8*Δ cells (Figs. 6 and 8). Thus, the PTS1 of Pex8p is dispensable for function and peroxisomal targeting of the protein. This is in support of previous observations that deletion of the putative C-terminal PTS1 of YlPex8p or HpPex8p abolishes neither the peroxisomal targeting nor the function of these proteins (22, 24). In addition, for HpPex8p it was shown that the N-terminal 16 amino acids comprise a functional PTS2 (22). In analogy, the amino acids 103–111 of ScPex8p contain a sequence motive that resembles a PTS2 consensus sequence, and, moreover, the N terminus of Pex8p (amino acids 1–112) alone is sufficient to direct a reporter protein into the peroxisomal matrix (data not shown). Accordingly, also the ScPex8p contains two potentially redundant targeting signals, a C-terminal PTS1 as well as an N-terminal one. Previous studies indicated that a buried PTS2 signal can bind to the PTS2 receptor, even though it is not located to the extreme N terminus (47, 61). However, we could not detect any interaction between the PTS2 receptor Pex7p of *S. cerevisiae* and either ScPex8p or HpPex8p.[2] Thus, a more detailed analysis needs to be carried out to demonstrate in either case that the N-terminal targeting signal is indeed a PTS2.

Data obtained by two-hybrid analysis and co-immunoprecipitation experiments show that Pex8p interacts with Pex5p. This observation was not surprising considering the presence of a PTS1 within Pex8p. However, we found that this interaction is not affected by deletion of the SKL of Pex8p, whereas a deletion of the SKL of Luciferase, a model PTS1 protein, abrogates its binding to Pex5p (Figs. 9 and 10). Moreover, C-terminal truncations in Pex5p abolish its interaction with PTS1 cargo proteins, but an interaction between the truncated Pex5p and Pex8p is still detectable although reduced in its strength (Fig. 9). Taken together, these findings argue for the presence of at least two binding sites for Pex5p within Pex8p. The strong interaction between Pex8pΔSKL and Pex5p could account for the efficient complementation by this truncated protein because it has to be considered that SKL-independent transport of Pex8p via the PTS1 receptor may still occur. However, the presence of a second binding site for Pex5p suggests that the Pex5p/Pex8p interaction does not simply reflect a targeting signal recognition but that the Pex5p/Pex8p association might be of importance for the function of both proteins in PTS1-dependent protein import into peroxisomes.

What might be the molecular function of Pex8p? Our data on ScPex8p as well as published data on Pex8p orthologues (22–24) suggest that it is an intraperoxisomal protein that is involved in protein import of both PTS1 and PTS2 proteins into peroxisomes. The existence of an intraperoxisomal binding partner for the predominantly cytosolic PTS1 receptor would be in agreement with the extended shuttle model of peroxisomal protein import, which suggests that the import receptors enter the peroxisome, release their cargo in the peroxisomal lumen, and shuttle back to the cytoplasm. However, the intracellular site of association between Pex5p and Pex8p remains to be determined.

With regard to a function for Pex8p in peroxisomal protein import, it is interesting to note that the initially selected *pex8* mutant of *P. pastoris* is characterized by a selective import defect for PTS1 proteins but still correctly localizes the PTS2 protein thiolase to the peroxisomal matrix (23). However, in all species including *P. pastoris*, deletions of the entire *pex8* gene lead to a complete import block. These observations suggest

[2] P. Rehling, A. M. Veenhuis, W.-H. Kunau, and R. Erdmann, unpublished observation.

that Pex8p might show two separate specificities for imported proteins, in agreement with separable functions related to either receptor or signal binding. Our observation that receptors and cargo still reach the peroxisomal translocation machinery (Fig. 11) suggests that this Pex8p-dependent step follows the docking event. The shuttle model of peroxisomal protein import requires the release of cargo at one step of the import process as well as the subsequent recycling of receptors. It can be speculated that Pex8p might be involved in one of these subsequent steps of the peroxisomal protein import pathway.

However, considering the strong requirement for Pex8p in import into peroxisomes and its own intraperoxisomal localization and therefore its own obligatory import into the organelle, a discrepancy becomes obvious. How can a *PEX*8 deleted cell ever be complemented with a plasmid encoding Pex8p that has to reach the peroxisomal lumen to function? We have to predict that ghosts of *pex8*Δ cells have a low import efficiency and that small amounts of imported Pex8p will subsequently increase the capability of the organelle to import other molecules. This hypothesis would be in agreement with a role of Pex8p in PTS receptor recycling.

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